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(57) Abstract

The present invention relates to a method of detecting in DNA obtained from an individual an abnormality in DNA which is associated with a genetic desease, with particular reference to cystic fibrosis, using oligonucleotide probes. The probes are labeled with two different fluorophores and hybridize to the regions of normal DNA which correspond to a region of DNA where an abnormal nucleotide sequence exists in a gene, such as that associated with that of cystic fibrosis. Hybridization is detected by fluorescence resonance energy transfer (FRET).

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DIAGNOSING CYSTIC FIBROSIS AND OTHER GENETIC DISEASES USING FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

Background of the Invention

Cystic fibrosis (CF) is the most common genetic disorder affecting the white population. The clinical manifestations of the disease include chronic pulmonary disease, pancreatic enzyme insufficiency and elevated sweat electrolytes. Patients with CF usually succumb to the pulmonary disease by the second decade of life. Increasingly, patients are surviving into adult life, albeit with pulmonary and gastrointestinal problems. The prognosis of CF depends entirely on its severity, age at first diagnosis and effective management of many complications.

Abnormally high electrical potential differences have been detected across the epithelial surfaces of CF exocrine tissues. The fundamental defect has been associated with decreased chloride ion conductance across the apical membrane of epithelial cells. Although progress has been made in the isolation of polypeptide components of an epithelial chloride channel that mediates conductance, the relationship of the channel to the clinical symptoms of CF has yet to be established. Despite extensive research efforts, the basic biochemical defect remains unknown.

The incidence of CF in North American whites is approximately 1 in 25,000 births. This suggests a gene frequency of 1 in 50 and a carrier frequency of

about 1 in 25. The defective gene was linked to a DNA polymorphism in 1985, and localized to the long arm of human chromosome 7. Subsequently, segments of DNA closer to the cystic fibrosis gene were identified, and DNA polymorphisms with particular alleles frequently associated with the cystic fibrosis (i.e., linkage disequilibrium) were reported. The cloning of the CF gene has been reported, and a three-base (three-nucleotide) deletion that removes phenylalanine 508 from the 1480 amino acid coding region was identified as the mutation that causes CF in the majority of cases.

Historically, the diagnosis of cystic fibrosis has been based on clinical findings and the biochemical abnormalities in sweat. An increase in sweat electrolytes, accompanied by one or more major clinical features, was the basis for diagnosis. Prenatal diagnosis and carrier detection were not possible.

Summary of the Invention

The present invention relates to a method of detecting in DNA obtained from an individual an abnormality in DNA, such as that associated with CF. In particular, it relates to a method of detecting in DNA obtained from an individual, a three-nucleotide or triplet deletion from the CF gene which has been shown to be associated with CF in approximately 70% of all cases. The present invention further relates to probes (DNA or RNA) useful in the method, and applicable to other genetic diseases in which a

deletion, insertion, modification or substitution of one or more bases in the primary structure of the normal DNA occurs.

The method of detecting an abnormality in a cystic fibrosis gene which is associated with or causative of cystic fibrosis relies on fluorescence resonance energy transfer (FRET) and can be carried out in DNA obtained (prenatally or postnatally) from an individual suspected of having or likely to develop cystic fibrosis or in DNA obtained from an individual thought to be a carrier.

In the method of the present invention, a pair of fluorophore-labeled oligonucleotide probes is combined with DNA obtained from an individual. The fluorescence energy transfer technique as related to homologous sequences was suggested by Heller and Morrison, in Rapid Detection and Identification of Infectious Agents, Eds. Kingsbury, J.J. and S. Falkau (Academic, New York,) pp. 245-256, and shown to be applicable to localization of specific sequences of DNA or RNA by Cardullo et al., Proc. Natl. Acad. Sci. USA, 85:8790-8794 (1988). The probes are complementary to the region of normal DNA which corresponds to a region of DNA where an abnormal nucleotide sequence exists in a gene associated with or causative of cystic fibrosis. Hybridization of both labeled oligonucleotide probes to DNA obtained from the individual occurs only to normal DNA and is detected by measuring fluorescence resonance energy transfer.

In particular, the invention relates to a method which makes use of a pair of fluorophore-

labeled oligonucleotide probes. One probe is comprised of a nucleotide sequence complementary to a region of DNA which is adjacent to an abnormal nucleotide sequence in a defective gene associated with or causative of cystic fibrosis. A second probe is labeled with a different fluorophore (i.e., from that present on the other probe) and is comprised of a nucleotide sequence complementary to the region of normal DNA corresponding to the region from which, in abnormal DNA, the three nucleotides are deleted and a region of DNA directly adjacent to the region, but in the opposite direction from that of the other probe. The fluorophores are covalently bound to the probes and are positioned on each probe such that when both probes are hybridized to an uninterrupted segment of normal DNA, the fluorophores will lie adjacent to one another.

Both probes will hybridize to DNA from a normal individual and the energy of the fluorophore (donor) of one probe will transfer to the fluorophore (acceptor) of the second probe. When both probes are hybridized to an uninterrupted segment of normal DNA, there will be an area between the probes lacking hybridization of complementary nucleotides, which provides the space for the fluorophores to lie adjacent to one another and transfer energy. This can be detected by fluorescence resonance energy transfer (FRET) by measuring the decrease in fluorescence (quenching) of the donor fluorophore and the increase in fluorescence of the acceptor fluorophore. If the energy transfer occurs, it is indicative of a normal gene or nucleotide sequence.

The probe which includes a nucleotide sequence complementary to the region of normal DNA that corresponds to the abnormal nucleotide sequence of a defective gene will not hybridize with DNA from an individual with cystic fibrosis. Consequently, there will be no energy transfer between fluorophores and no increase and decrease in fluorescence of the fluorophores occurs. The absence of energy transfer is, thus, indicative of cystic fibrosis. Further, the confirmation of the presence of the CF gene can be carried out through the use of probes specific for the CF gene.

The present invention offers several advantages over existing technologies. Current methods of detecting the absence or presence of abnormal nucleotide sequences related to cystic fibrosis usually involve the burdensome task of immobilizing, onto a solid support, the oligonucleotide probes used for capturing the nucleotide sequence of interest or sample nucleotide sequences to be tested. This is needed because current testing methods require the separation of hybridized nucleotide sequences from non-hybridized sequences. The present invention does not require these separation steps, and consequently, immobilization of probes or sample nucleotide sequences is unnecessary. In addition, the use of solid supports is usually accompanied by problems of nonspecific binding of detector oligonucleotide probes to the solid support, resulting in inaccurate determinations of hybridization between the nucleotide sequence of interest and detector probes.

Lastly, the present method does not require the use of radioactivity, and consequently, its troublesome disposal. In summary, the present invention is an accurate and sensitive method of detecting abnormal nucleotide sequences in the cystic fibrosis genome, which is also convenient and safe.

Brief Description of the Drawings

Figure 1 is a schematic representation of the hybridization of fluorophore-labeled nucleic acids complementary to distinct, but closely spaced, sequences of a longer unlabeled nucleic acid.

Figure 2 is a schematic representation of one embodiment of the present invention, illustrating the use of paired fluorophore-labeled probes complementary to normal DNA wherein 1) the hybridization of both fluorophore-labeled probes with normal DNA and the resulting fluorescence energy transfer; and 2) the lack of hybridization with CF DNA of the fluorophore-labeled probe containing the nucleotide sequence complementary to the region of normal DNA corresponding to the trinucleotide deletion and adjacent 5' nucleotides of cystic fibrosis DNA and the lack of fluorescence energy transfer.

Figure 3 is a schematic representation of one embodiment of the present invention, illustrating the use of paired fluorophore-labeled probes complementary to CF DNA and showing 1) the hybridization of both fluorophore-labeled probes with CF DNA and the resulting fluorescence energy transfer; and 2) the lack of hybridization with normal DNA of the

fluorophore-labeled probe containing the nucleotide sequence complementary to the region of CF DNA corresponding to the trinucleotide deletion and adjacent 5' nucleotides of cystic fibrosis DNA and the lack of fluorescence energy transfer.

Figure 4 is a graphic representation of the modulation of fluorescence intensity upon 8-mer hybridization at fixed numbers of donor molecules and increasing concentration of the complementary oligonucleotides.

Figure 5 is a graphic representation of the transfer efficiency of fluorescein and rhodamine attached to the 5' ends of complementary oligonucleotides of various lengths.

Figure 6 is a graphic representation of changes in fluorescence intensity of donor and acceptor-linked 8-mers as a function of temperature.

Detailed Description of the Invention

The present invention is a method of detecting, in DNA or RNA obtained from an individual, a defective gene(s) associated with or causative of cystic fibrosis. The method is useful in diagnosing cystic fibrosis in an individual and in determining whether an individual is a carrier of the defective gene(s). The method utilizes a pair of fluorophore-labeled oligonucleotide probes complementary to the region of normal DNA which corresponds to the region of DNA from which, in DNA associated with or causative of cystic fibrosis, three nucleotides are deleted (i.e., the region in which an abnormal nucleotide sequence occurs). The present method

relies on means of detection of fluorescence resonance energy transfer as an indicator of the presence or absence of abnormal DNA.

Hybridization of two separate segments of DNA (i.e., oligonucleotide probes) to adjacent regions of a third complementary single strand may be detected by non-radiative fluorescence resonance energy transfer, provided that two fluorophores with overlapping excitation and emission spectra are attached to the hybridizing segments of DNA and, once they are hybridized, the distance between the two fluorophores they bear is appropriate for FRET to occur. For example, the 3' end of one oligonucleotide can have a fluorescein covalently attached, and the 5' end of the other oligonucleotide can have a rhodamine covalently attached. This can be carried out, or example, a described in Agrawal, S., et al., Nucleic Acids Res., 14: 6227-6245 (1986); Tet Lett., 31:1543-1546 (1990); Nucleic Acids Res., 18:5419-5423 (1990) and Emson, P.C., et al., Methods in Enzymology, 168:753-761 (1988). The teachings of both of these references are incorporated herein by reference. The excitation and emission spectra for the fluorophores used for labeling the two probes must overlap. With the two fluorophores mentioned above, the excitation wavelength for fluorescein (472 nm) will excite an emission wavelength of the rhodamine at 577 nm. An important consideration in selecting or designing probes or oligonucleotide sequences is the distance which will separate them once each hybridizes to the region of the longer DNA sequence to which it is

complementary. In general, the closer the two fluorophores are to one another, the greater will be the energy transfer. Maximal separation is approximately 50-75 angstroms. Thus, a distance represented by 4-6 bp for two segments of DNA hybridizing to a third complementary uninterrupted segment would represent an acceptable proximity of the fluorescein and rhodamine fluorophores. The acceptable basepair distances between other pairs of fluorophores can be determined experimentally by one skilled in the art.

One embodiment of the present invention relates to the detection of the segment of DNA from a cystic fibrosis gene in which the genetic deletion (-TTT-) occurs on chromosome 7. The detection of this deletion can be carried out as follows: Two probes are used: one probe (a first probe) which is approximately 20 oligonuclotides in length, which is complementary to the cystic fibrosis gene and 5' to the deletion, and labeled with fluorescein (e.g., by attaching fluorescein by a linker to the 5' end of the oligonucleotide DNA sequence) and another probe (a second probe) which is a hexamer complementary to the nucleotide sequence of the -TTT- deletion and the adjacent three nucleotides in the 3' direction and is labeled with rhodamine attached to its 3' end.

Exon 10 of chromosome seven, as described by Reardon, J.R., et al., (Science, 245:1066-1073 (1989)) is isolated from a normal genome and from a cystic fibrosis genome. Using conditions detailed in Cardullo, R.A., Proc. Natl. Acad. Sci., USA, 85: 8790- 8794 (1988), the components to be hybridized

are combined: 1) Exon 10, 2) a 20-mer complementary to the GGC-ACC-ATT-AGA-GAA-AAT-AT portion of the gene and bearing a 3'-terminal fluorescein molecule, and 3) a hexamer complementary to TTT-GGT and bearing a 5'-terminal rhodamine molecule. There will be two forms of Exon 10: one from a normal genome and a second from a cystic fibrosis genome. The reagents or components are combined at a temperature appropriate for hybridization of oligonucleotides of the length used to occur (e.g., 20°C). At this temperature, during the hybridization reaction, the fluorescein is excited using a laser beam of approximately 472 nm. With the 20-mer hybridized to the specific sequence of the genome, the fluorescein and rhodamine will be in close enough proximity to permit the fluorescence energy transfer. Thus, a rhodamine emission around 577 nm will occur in the normal genome segment.

As illustrated in Figure 2, the rhodamine-AACCA hexamer will be hybridized to the -TTTGGT- segment of the normal genome. This results in energy transfer from fluorescein to rhodamine and fluorescence emission from rhodamine. In the case in which DNA being analyzed is altered (i.e., in this case, contains the trinucleotide deletion), as also illustrated in Figure 2, there is no -TTT- trinucleotide, due to the genetic deletion. Hybridization of the rhodamine-labeled hexamer (rhodamine-AAA-CCA-5') with the segment of the cystic fibrosis genome missing the -TTT- cannot occur. At the hybridization temperature chosen (e.g., 20°C), the three members of the probe

complementary to chromosomal DNA will not hybridize effectively with a complementary trimer.

Therefore, Exon 10 from a normal gene will give a rhodamine fluorescence energy transfer, while Exon 10 from a cystic fibrosis gene with a -TTT- deletion will fail to give the rhodamine fluorescence when subjected to the nucleic acid hybridization procedure described above.

As illustrated in Figure 3, detection of the cystic fibrosis defect is accomplished by employing the labeled probe 5'-ACCGAT, completely hybridizable to the sequence 5'-ATCGGT of the cystic fibrosis genome. In the case of the normal genome, however, the ATCGGT encounters a mismatch and does not hybridize at 20°C. The FRET energy is, therefore, not transferred from fluorescein to rhodamine and the rhodamine emission spectrum is not activated. The probes may be longer than a hexanucleotide.

The detection of any known defect in a nucleotide sequence of a gene(s) relating to cystic fibrosis can be achieved using a pair of two oligonucleotide probes: 1) a first probe complementary to an area of DNA adjacent to the known defect in the nucleotide sequence of a gene(s); and 2) a second probe complementary to: a) a region of normal DNA corresponding to the region in which the known defect in the nucleotide sequence of these genes occurs, and b) a region adjacent, in the opposite direction of the first probe, to the defect-containing region. Each probe is labeled with a fluorophore and the fluorophores on the two probes are different from one another. The fluorophores

can be attached to the 3' or 5' end of either probe; however, the attachment of the fluorophores must be matched in such positions that when hybridization occurs between the two probes and an uninterrupted segment of DNA, the two fluorophores of the probes are adjacent to one another. When both probes are hybridized to an uninterrupted segment of normal DNA from a sample, there will be an area between the two probes which lacks hybridization of complementary nucleotides. In this area, the fluorophores of the two probes lie adjacent to one another. The distance between the two fluorophores must be sufficiently close to allow the efficient transfer of energy between the two fluorophores but not so close as to cause steric hinderance between the two probes upon hybridization. The excitation and emission spectra of the different fluorophores must overlap to achieve energy transfer. One fluorophore acts as an energy donor and the other acts as an energy acceptor. When in proximity to one another, which occurs with hybridization of both probes, the fluorescence of the energy donor decreases as the fluorescence of the energy acceptor increases. Examples of such fluorophores are fluorescein and rhodamine.

Probes useful in the present method can be made using genetic engineering techniques or can be synthesized chemically, such as by the phosphoramidite method using a commercial DNA synthesizer and β-cyanoethyl phosphoramidite.

Preparation of fluorescently labeled oligonucleotide probes can be achieved by derivatization of

the desired end to be labeled and subsequent attachment of the fluorophore. Briefly, for 5' fluorophore labeled oligonucleotide probes, an aminohexyl linker can be introduced onto the 5' end of the oligonucleotide by the use of an extra cycle of phosphoramidite synthesis (9-fluorenyl) methooxycarbonylaminohexyl β-cyanoethyl MN-diisopropylamino phosphite in the coupling reaction as described by Agrawal, S. et al., supra, and Emson, P.C. et al., supra. After removal of protective groups with concentrated ammonia solution, the aminohexyl linked oligonucleotide can be purified by reverse- phase high pressure liquid chromatography (HPLC).

The 3' end derivatization of oligonucleotides with an amino group can be based on established chemistry for 3' end labeling of RNA, such as that described in Zamecnik, P.S. et al., Proc. of the Natl. Acad. Sciences USA, 46:811-822 (1960); Booker, T.R. et al., Nucleic Acids Res., 5:363-384 (1978), the teachings of which are hereby incorporated by reference. To adapt this chemistry for labeling DNA, synthesis of the desired oligonucleotide sequence can be carried out on 5'-dimethoxytrityl--3'(2')-acetylribonucleoside2'(3')- linked to long-chain alkylamino controlled-pore glass support (20mM/gm). After the synthesis, protecting groups can be removed in concentrated ammonia. Crude oligonucleotides can then be oxidized with pyridate, reacted with 1,6-diaminohexane, and reduced by sodium cyanoborohydride as described in Agrawal, S. et al. and Booker, T.R. et al. The amino-oligonucleotides can be purified by reverse phase HPLC

because they are retarded to a significantly greater extent than underivatized oligonucleotides.

Attachment of fluorescein, using fluorescein isothiocyanate, or tetramethyl rhodamine, using tetramethyl rhodamine isothiocyanate, to the derivatized oligonucleotides and subsequent purification can be carried out according to the procedures described in Agrawal, S. et al. and Emson, P.C. et al., supra.

The present method can include the following steps: 1) obtaining from an individual a sample to be analyzed; 2) treating the sample to render nucleic acids present available for hybridization with complementary nucleotide sequences; 3) combining the treated sample and a pair of appropriate fluorophore-labeled oligonucleotide probes, under conditions appropriate for hybridization of complementary sequences to occur; and 4) determining whether fluorescence resonance energy transfer occurs. A lack of energy transfer is indicative of cystic fibrosis.

The present method can be used on DNA from a variety of tissues. For example, a sample can be obtained prenatally by amniocentesis or postnatally by surgical biopsy. Once obtained, the sample is treated in such a manner that the nucleic acids present in the sample are available for hybridization with complementary nucleic acid sequences, which are the selected oligonucleotide probes described above. For example, a sample can be treated with an agent which disrupts the cellular and molecular structures of the tissue. Cells can

be disrupted using chaotropic agents which disrupt the molecular structure of the tissue. That is, the agent denatures the secondary, tertiary and/or quarternary structures of biopolymers, including proteins, nucleic acids, polysaccharides which are generally found in biological specimens. Examples of chaotropic agents include chaotropic salts (e.g., guanidinium thiocyanate), hydrolytic enzymes (e.g., proteases) and compounds that disrupt hydrophobic interactions (e.g, sodium dodecylsulfate, phenols, dimethylformamide, dimethylsulfoxide, tetramethyl-urea or guanidinium hydrochloride. Physical or mechanical means of disrupting molecular structures (e.g., bead beading and sonication) can be used to release nucleic acids. If necessary, nucleic acids present in the tissue sample and released from it can be treated further to ensure that they are available for hybridization with complementary nucleic acid sequences (e.g., by heating to render double stranded sequences single stranded). Agents and techniques that disrupt molecular structures can be used singly or in various combinations for this purpose.

After the nucleic acids are rendered available for hybridization, the sample is combined with a pair of oligonucleotide probes as described above, which hybridize selectively to the region of normal DNA which corresponds to the region of DNA where a known defect exists in the nucleotide sequence(s) of a gene(s) associated with or causative of cystic fibrosis.

The optimum temperature for hybridization of both oligonucleotide probes to sample nucleic acids will depend on the nucleotide length of both probes and can be determined experimentally by someone skilled in the art. Figure 6 illustrates the melting temperature (T_m) for hybridization of unmodified oligonucleotides, and of oligonucleotides with modifications at the internucleoside phosphates. A 20-mer unmodified deoxyoligonucleotide has a T_m of 66°C; while a trimer unmodified deoxyoligonucleotide has a T_m of approximately 5°C.

The method of the present invention can be carried out in such a manner that hybridization occurs in an aqueous environment without the need for a solid support. The treated sample is present in a liquid preparation, such as a phyiological salt solution. The oligonucleotide probes are also present in a liquid preparation. The two preparations are combined, to produce a sample-probe combination. This results in contact between nucleic acid sequences present in the sample, and the oligonucleotide probes. If nucleotide sequences which are complementary to the selected set of nucleic acid probes are present, hybridization will occur.

Detection of hybridization is carried out by exposing the sample to a wavelength appropriate for excitation of the donor fluorophore. For example, if the donor fluorophore is fluorescein, a wavelength of 472 nm is used. The energy from the excited fluorescein is transferred to the acceptor fluorophore, such as rhodamine. The fluorescence

emission wavelength of the acceptor fluorophore is then measured. Typically, the background fluorescence intensity of phosphate-buffered saline solution is determined. To this solution, quantities of donor labeled or unlabeled oligonucleotides in phosphate-buffered saline are added in steps and the fluorescence intensity is determined. Oligonucleotides containing acceptor fluorophores are then added in volume steps. Energy transfer is observed by both quenching and acceptor enhancement. Transfer efficiencies are determined from the quenching data. This involves correcting the data for dilution and for quenching by unlabeled complement. Thus, if $Q_{d,u}$ and $Q_{d,a}$ are the quenching observed for unlabeled and labeled complements, the transfer efficiency is given by $E_t=(Q_{d,a}-Q_{d,u})/(1-Q_{d,u})$. Acceptor labeled oligonucleotides are added until E_t is constant. The degree of quenching of the donor fluorophore and excitation emission of the acceptor fluorophore are determined for each sample and compared. Lack of hybridization between oligonucleotide probes and sample nucleic acids is detected by the absence of quenching of the donor fluorophore and absence of an enhanced emission spectra of the acceptor fluorophore. Normal samples and samples obtained from individuals suspected of having cystic fibrosis or being a carrier of a cystic fibrosis gene are compared.

The FRET technique can be used for the diagnosis of other cellular diseases involving DNA or RNA, in which nucleotide deletions, changes or additions occur. For example, Familial Hypertrophic

Cardiomyopathy in the β cardiac MHC gene, exon 27 has a replacement of an alanine by a serine residue at one position. T in a codon takes the place of an A, thus, converting the coding sequence from GCC(ala) to TCC(ser) (Tanigawa, G. et al., Cell, 62:991-998 (1990)).

In Myoclonic Epilepsy and Ragged-Red Fiber Disease (MERRF) a mutation occurs at nucleotide pair 8344 in the mitochondrial DNA in the tRNA lys TΨC loop, with an A-to-G transition mutation (Shoffner, J.M. et al., Cell, 61:931-937 (1990)).

In Albright's Hereditary Osteodystrophy, the $G_s\Psi$ gene, contains in exon 1 an A-to-G transition at position +1 in the $B_s\alpha$ allele. This mutation converts the initiator ATG (methionine) codon to GTG (valine), thus, blocking initiation of translation at the normal site (Patten, J.L. et al., N.E.J. Med, 322:1412-1419 (1990)).

In the disease characterized by a deficiency of lipoprotein lipase (LPL) activity, known as $LPL_{Bethesda}$, there is a single base substitution, G → A, at position 781 in the fifth exon, which results in an Ala → Thr substitution at residue 176 of LPL (GCA becomes ACA) (Beg, O.U. et al. Proc. Natl. Acad. Sci. USA, 87:3474-3478 (1990)).

In the disease known as Achondrogenesis, there is a heterozygous single exon deletion in the type II procollagen gene (COL2A1). A single base change, G → A occurs in exon 51 of the RFLP (+) allele and results in a glycine to serine substitution at amino acid position 191 in the C-propeptide of type II procollagen. A second substitution occurs at the

100th nucleotide of exon 46, and converts the normal glycine codon at position 943.(GGC) to serine (AGC) (Vissing, H. et al., J. Biol. Chem., 364:18265-18267 (1989)).

In Tay-Sachs Disease (G_{M2} gangliosidosis, type 1) three point mutations have been identified (for a review, see Triggs-Raine, B.L. et al., N.E.J. Med., 323:6-12 (1990). In one of these a four base pair insertion occurs in exon 11, accounting for approximately 70 percent of the cases of infantile Tay-Sachs disease in Ashkenazi Jews (Myerowitz, R. and F.C. Costigan, J. Biol. Chem., 263:18567-18569 (1988)).

In hemophilia A, the codon for arginine (CGA) at amino acid 2135 is mutated to the stop codon TGA (Youssoufian, H. et al., Nature, 324:380-382 (1986)).

In sickle cell anemia in the B^S-globin chain, there is a substitution of A for T at codon 6. This mutation changes the codon GAG (Glu) to GTG (Val) (Antonarakis, S.E. et al., Hum. Genet., 69:1-14 (1985)).

A list of 16 diseases in which point mutations have been identified is given in a review of genetic disorders at the DNA level by S.E. Antonarakis (Antonarakis, S.E., N.E.J. Med., 320:153-163 (1989)). In addition to those mentioned specifically above are Gaucher's disease hypobetalipoproteinemia, Osteogenesis imperfecta associated with a frameshift mutation, Gyrate atrophy, Diabetes mellitus due to abnormal insulins, Hereditary persistence of fetal hemoglobin,

Phenylketonuria, α_1-Antitrypsin deficiency, α-Thalassemia, Familial hypercholesterolemia, Ornithine transcarbamylase deficiency, Hemophilia B and Hemophilia A.

In principle, wherever a point mutation has been identified in a genome, it presents an opportunity to compare the hybridization Tms of small segments of a normal genome and of a disease altered genome by means of the FRET technique, and to diagnose the point mutation by an alteration in the Tm of the affected as compared with the normal genome segment.

When a single base deletion, alteration or insertion occurs in the diseased state, the hybridization association difference between the perfectly matched hybrids and those with mismatch(es) can be magnified by lowering the hybridization temperature. Thus, for example, in the early experiments defining the particular trinucleotides coding for individual amino acids, a hybridization temperature around 4°C was used, in order to induce effective hybridization with trinucleotides. At 37°C a trinucleotide will not hybridize effectively with its perfectly matched base complement.

The invention is further illustrated by the following specific examples, which are not intended to be limiting in any way.

EXAMPLE 1 Effect of Acceptor Concentration on Transfer Efficiency

The teachings of all scientific publications cited in all examples herein are hereby incorporated by reference.

Fluorescence measurements were made in a Perkin-Elmer spectrofluorimeter equipped with a temperature controlled chamber and Glan-Thompson polarizer. The excitation wavelengths used for fluorescein and acridine orange were 472 nm and 503 nm, respectively. The emission wavelengths used for fluorescein, acridine orange and rhodamine were 517 nm, 522 nm and 577 nm, respectively.

The background fluorescence intensity of 85 μl of phosphate-buffered saline (PBS: 0.138 m NaCl/0.01 m phosphate, pH 7.2) in a 200 μl quartz cuvette (optical solution path length = 0.3 cm) was determined. To this cuvette, 15 μl of approximately 5 mM donor-labeled or unlabeled oligonucleotides in PBS was added in 5 microliter steps and the intensity was determined. Oligonucleotides containing acceptor fluorophore was then added in 5 μl steps. Energy transfer was observed by donor quenching and acceptor enhancement. Transfer efficiencies were determined from the quenching data. This involved correcting the data for dilution and for quenching by unlabeled complement. Inner filter effects were negligible. Thus, if $Q_{d,u}$ and $Q_{d,a}$ are the quenching observed for the nonlabeled and labeled complements, the transfer efficiency is given by the following equation:

$$E_t = (Q_{d,a}-Q_{d,u})/(1-Q_{d,u})$$

Acceptor-labeled oligonucleotides were added until E_t was constant. Experiments were performed at 5°C.

To determine the maximum efficiency of transfer between donor and acceptor fluorophores attached to oligonucleotides, the emission spectrum of acceptor was followed as a function of increasing acceptor concentration at a fixed number of donor molecules. The first experiments were performed using two complementary oligonucleotides with donor and acceptor fluorophores attached at either end of the hybridized complex. Attachment of fluorescein, using fluorescein isothiocyanate, or tetramethylrhodamine using tetramethylrhodamine isothiocyanate, to the derivatized oligonucleotides and subsequent purification were carried out according to the methods of Agrawal, S. et al., supra, and Emson, P.C., supra. One oligonucleotide had fluorescein attached to its 5' end (donor) whereas the other complementary nucleotide had rhodamine attached to its 5' end (acceptor). Quenching and transfer efficiency were determined for oligonucleotides containing 8 nucleotides, 12 nucleotides and 16 nucleotides.

As shown in Figure 4, emission spectra are presented as a function of increasing rhodamine-linked 8-mer concentration to a fixed number of fluorescein-linked 8-mer molecules. As the amount of rhodamine-linked 8-mer was increased, there was a decrease in fluorescein emission intensity (517 nm) and an increase in rhodamine emission intensity (577 nm). Saturation of both the fluorescein quenching and the rhodamine enhancement

occurred when the ratio of acceptor to donor exceeded 2:1. The maximum quenching of fluorescein upon saturation was 0.63 in the presence of donor and acceptor. When the experiment was repeated with fluorescein-linked oligonucleotides and its unlabeled complement, fluorescein emission intensity was quenched 0.26 from its maximum value with no detectable increase in intensity at 577 nm, as shown in Figure 4. Thus, fluorescence was modulated in three ways upon hybridization: a decrease in fluorescein emission upon binding to an unlabeled complementary oligonucleotide, a larger decrease in fluorescein emission intensity upon binding to a rhodamine-linked complementary oligonucleotide, and the detection of rhodamine emission intensity upon binding to a rhodamine-linked complementary oligonucleotide. The first phenomenon represents a quenching of the fluorophore upon binding to its unlabeled complement, while the latter two phenomena represent modulation of fluorescence intensity due to energy transfer. The degree of fluorescein quenching due to energy transfer alone was calculated from the above equation. In the case of the 8-mer, the transfer efficiency between fluorescein and rhodamine was, therefore, about 0.5.

Comparable experiments using 12-mers and 16-mers were also performed, the results of which are shown in Table 1. In general, the amount of quenching in the absence of acceptor was independent of chain length and had a value of 0.26 ± 0.02 for all oligonucleotides (mean ± -SD for 4 determinations of each n-mer, where n = 8, 12, or 16

nucleotides). In the presence of rhodamine-linked complementary oligonucleotides, the degree of fluorescein quenching due to energy transfer alone decreased with increasing chain length. As shown in Figure 5, hybridization was complete for all three chain lengths at an acceptor/donor ratios, no modulation in the corrected fluorescein or rhodamine signal was observed. Subsequent experiments using these oligonucleotides were done at an acceptor/donor ratio of 4:1 to ensure that hybridization was complete.

Table 1. Modulation of fluorescein intensity at saturating levels of ODNT with and without rhodamine attached for ODNTs of chain length *n*

n	$q_{f,f}$	$q_{f,u}$	E_t	R/R_0
8	0.632 ± 0.046	0.265 ± 0.021	0.501 ± 0.035	0.99 ± 0.02
12	0.423 ± 0.030	0.265 ± 0.013	0.215 ± 0.052	1.24 ± 0.05
16	0.295 ± 0.017	0.262 ± 0.013	0.045 ± 0.018	1.66 ± 0.10

Data represent mean ± SD for four different experiments. See Eqs. 1 and 2. Subscripts f, r, and u indicate fluorescein-labeled, rhodamine-labeled, and unlabeled ODNT.

EXAMPLE 2 Effect of Temperature on Transfer Efficiency

The effect of temperature on hybridization was also followed for different chain lengths (8, 12 and 16-mers) at saturating concentrations of acceptor-linked oligonucleotide. The resulting melting temperatures (T_m), defined as the midpoint values of fluorescein quenching or rhodamine enhancement over a temperature range of 0-60°C, were compared with absorbence values that are at 260 nm. Above 50°C, there was no fluorescein quenching nor detectable rhodamine signal. As the temperature was lowered, the fluorescein intensity decreased and the rhodamine intensity increased in a sigmoidal manner

as shown by (o) in Figure 6, indicating an increase in transfer efficiency with the rhodamine acceptor attached to the complementary 8-mer. This agreed well with the absorbence data, which showed a characteristic sigmoidal decrease in A_{260} with decreasing temperature, indicating hybridization of complementary nucleotides. In addition, there was a concurrent increase in rhodamine emission intensity (o).

In general, there was no significant difference between T_m values obtained by fluorescein quenching and by decreased A_{260} signal with decreasing temperature. The T_m values obtained by fluorescein quenching were 23.8 ± 4.2°C, 38.3 ± 4.5°C, and 47.2 ± 5.2°C for the 8-mer, 12-mer and 16-mer, respectively (mean ± SD for 4 determinations). By comparison, the T_m values obtained by a decrease in A_{260} were 24.5°C, 37.5°C, and 46.0°C (for the 8-mer, 12-mer and 16-mer, respectively). Hence, in all cases, the T_m determined by fluorescence was within 3% of the T_m determined by A_{260}.

EXAMPLE 3 Hybridization of Two Labeled Oligonucleotides to a Complementary Strand

Experiments were also performed with two fluorescently labeled oligonucleotides hybridized to a longer complementary strand as schematically illustrated in Figure 1. When these three strands hybridized, only 4 bases separated the fluorescein donor from the rhodamine acceptor. As in Example 1, quenching of donor fluorescence by energy transfer

increased to saturation with acceptor concentration. Table 2, line a, shows the results of these experiments. In the presence of fluorescein-labeled oligonucleotides and unlabeled oligonucleotides hybridized to the 29-mer, the quenching of fluorescein emission was about 0.27. In the presence of rhodamine acceptor, the quenching was enhanced to 0.71 and there was a large fluorescence signal at the rhodamine peak (577 nm). Hence, the transfer efficiency, given by the above equation in Example 1, was about 0.6.

Table 2. Quenching and transfer efficiency of two 12-mers attached to a 29-mer (line A) and of two hybridized 12-mers in the presence of acridine orange (line B)

	$q_{d,T}$	$q_{d,a}$	E_t	R/R_0
A	0.712 ± 0.024	0.276 ± 0.012	0.602 ± 0.017	0.933 ± 0.011
B	0.573 ± 0.027	0.109 ± 0.009	0.520 ± 0.016	—

Data represent the mean ± SD for four different experiments. Subscript d represents fluorescein in line A and acridine orange in line B.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiment of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

1. A pair of oligonucleotide probes, wherein:
   a) a first oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to a region of human chromosomal DNA adjacent to a region in which an alteration associated with cystic fibrosis occurs and is labeled with a fluorophore at the end which, upon hybridization to the region of human chromosomal DNA, is closer to the region in which the alteration occurs; and
   b) a second oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to the region of normal human chromosomal DNA which corresponds to the region in which the alteration associated with cystic fibrosis occurs and is labeled with a fluorophore at the end which, upon hybridization to the region of human chromosomal DNA, is closer to the first probe.

2. A pair of oligonucleotide probes, wherein:
   a) a first oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to a region of human chromosomal DNA adjacent to the 5' end of the region where the trinucleotide deletion of chromosome seven associated with cystic fibrosis

occurs and is labeled with a fluorophore at the 5' end; and

b) a second oligonucleotide probe comprised of a nucleotide sequence which hybridizes to the region of normal human chromosomal DNA which corresponds to the region where the trinucleotide deletion of chromosome seven associated with cystic fibrosis occurs and is labeled with a fluorophore at the 3' end.

3. A pair of oligonucleotide probes, each comprised of a nucleotide sequence and a covalently bound fluorophore:

a) 3'-CCG TGG TAA TCT CTT TTA TA FL-5'

b) 3'-FL AAA CAA-5', wherein the fluorophore (FL) covalently bound to one probe is a donor fluorophore and the fluorophore covalently bound to the other probe is an acceptor fluorophore.

4. A pair of oligonucleotide probes of Claim 3, wherein the donor fluorophore is fluorescein and the acceptor fluorophore is rhodamine.

5. A method of detecting in a sample a defective gene associated with or causative of a disease, comprising combining the sample, treated so as to render nucleic acids present in the sample available for hybridization with complementary oligonucleotide probes, under appropriate

conditions, with a pair of fluorophore-labeled oligonucleotide probes, wherein:

a) a first oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to a region of human chromosomal DNA adjacent to a region in which an alteration associated with said disease occurs and is labeled with a first fluorophore at the end which, upon hybridization to the region of human chromosomal DNA, is closer to the region in which the alteration occurs; and

b) a second oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to the region of normal human chromosomal DNA which corresponds to the region in which the alteration associated with said disease occurs and is labeled with a second fluorophore at the end which, upon hybridization to the region of human chromosomal DNA, is closer to the first probe; and separated by a distance which allows the efficient transfer of energy between the first and second fluorophore.

6. A method of Claim 5, wherein hybridization of complementary nucleotide sequences is detected by determining fluorescence resonance energy transfer.

7. A method of Claim 5 wherein, upon hybridization, the two oligonucleotide probes are separated optimally by a distance of two to four nucleotide bases, with longer and shorter distances being acceptable as long as energy transfer occurs.

8. A method of detecting in a sample a gene associated with or causative of cystic fibrosis, comprising combining the sample, treated so as to render nucleic acids present in the sample available for hybridization with complementary oligonucleotide probes, under appropriate conditions, with a pair of fluorophore-labeled oligonucleotide probes, wherein:

a) a first oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to a region of human chromosomal DNA adjacent to a region in which an alteration associated with cystic fibrosis occurs and is labeled with a first fluorophore at the end which, upon hybridization to the region of human chromosomal DNA, is closer to the region in which the alteration occurs; and

b) a second oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to the region of normal human chromosomal DNA which corresponds to the region in which the alteration associated with cystic fibrosis occurs and is labeled with a second fluorophore at the end

which, upon hybridization to the region of human chromosomal DNA, is closer to the first probe; and separated by a distance which allows the efficient transfer of energy between the first and second fluorophore.

9. A method of Claim 8 wherein hybridization of complementary nucleotide sequences is detected by determining fluorescence resonance energy transfer.

10. A method of Claim 8 wherein, upon hybridization, the two oligonucleotide probes are separated by a distance of two to four nucleotide bases.

11. A method of detecting in a sample a trinucleotide delation of chromosome seven associated with or causative of cystic fibrosis, comprising combining the sample, treated so as to render nucleic acids present in the sample available for hybridization with complementary oligonucleotide probes, under appropriate conditions, with a pair of fluorophore-labeled oligonucleotide probes, wherein:

a) a first oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to a region of human chromosomal DNA adjacent to a region in which said trinucleotide deletion associated with cystic fibrosis occurs and is labeled with

a first fluorophore at the end which, upon hybridization to the region of human chromosomal DNA, is closer to the region in which the alteration occurs; and

b) a second oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to the region of normal human chromosomal DNA which corresponds to the region in which the trinucleotide deletion of chromosome seven associated with cystic fibrosis occurs and is labeled with a second fluorophore at the end which, upon hybridization to the region of human chromosomal DNA, is closer to the first probe; and separated by a distance which allows the efficient transfer of energy between the first and second fluorophore.

12. A method of Claim 11 wherein hybridization of complementary nucleotide sequences is detected by determining fluorescence resonance energy transfer.

13. A method of Claim 11 wherein, upon hybridization, the two oligonucleotide probes are separated by a distance of approximately two to four nucleotide bases, with longer and shorter distances being acceptable as long as energy transfer occurs.

14. A method of diagnosing cystic fibrosis in an individual, comprising the steps of:

a) rendering nucleic acids present in a sample obtained from the individual available for hybridization with complementary oligonucleotide probes;

b) combining the product of step (a) with a pair of oligonucleotide probes, wherein:

i) a first oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to a region in which an alteration associated with cystic fibrosis occurs and is labeled with a fluorophore at the end which, upon hybridization to the region of human chromosomal DNA, is closer to the region in which the alteration occurs; and

ii) a second oligonucleotide probe is comprised of a nucleotide sequence which hybridizes to the region of normal human chromosomal DNA which corresponds to the region in which the alteration associated with cystic fibrosis occurs and is labeled with a fluorophore at the end which, upon hybridization to the region of human chromosomal DNA, is closer to the first probe;

c) detecting hybridization of complementary nucleotide sequences by determining fluorescence resonance energy transfer; and

d) comparing the fluorescence resonance energy transfer determined in step (c) with the fluorescence resonance energy transfer which occurs when hybridization takes place under the same conditions.

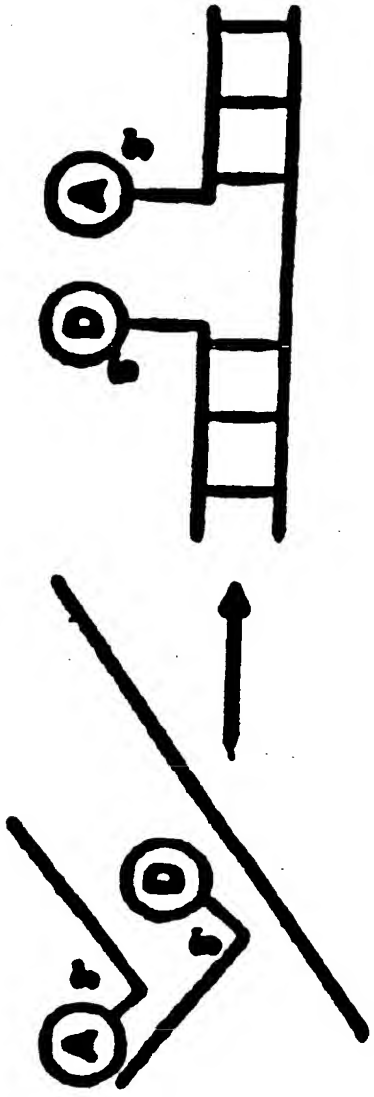


FIGURE 1

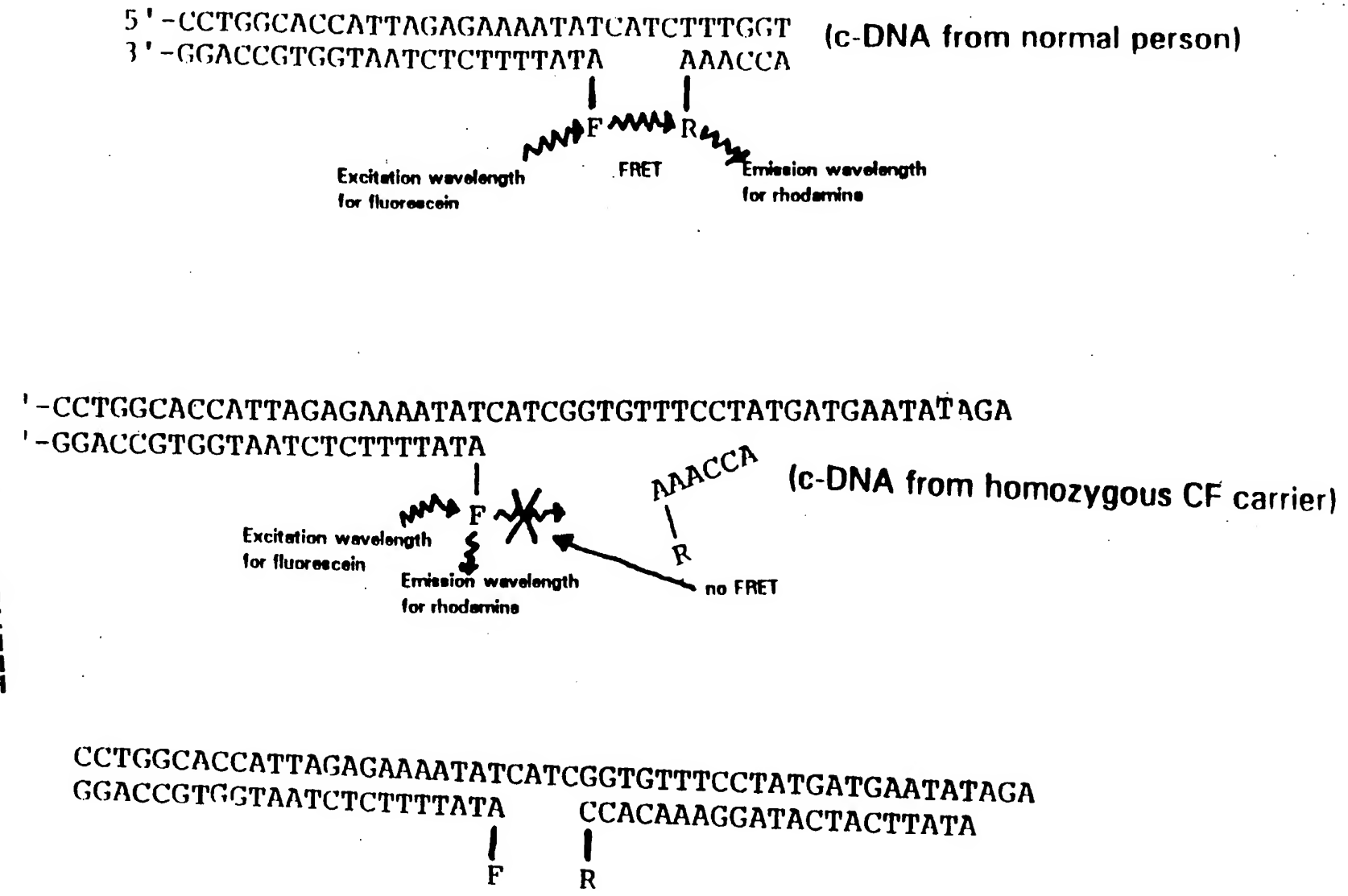
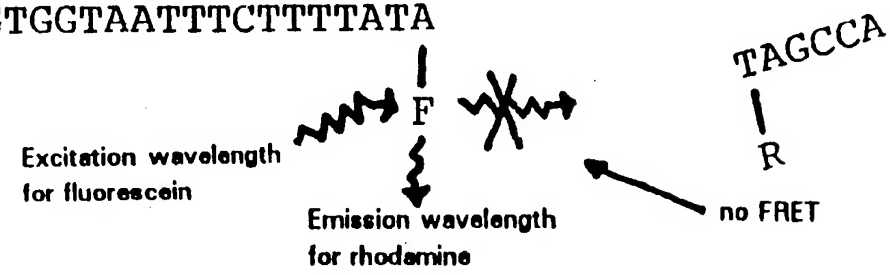


Figure 2

(c-DNA from normal pe

ATTATGCCTGGCACCATTAAAGAAAATATCATCTTTGGTGTTTCCTATGATGAATATAGA
TAATACGGACCGTGGTAATTTCTTTTATA



(c-DNA from homozygous CF carrie

5'-ATTATGCCTGGCACCATTAAAGAAAATATCATCGGTGTTTCCTATGATGAATATAGA
3'- TACGGACCGTGGTAATTTCTTTT TAGCCA

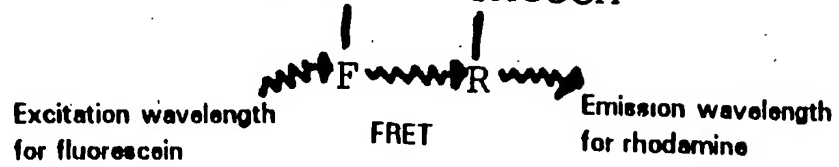


Figure 3

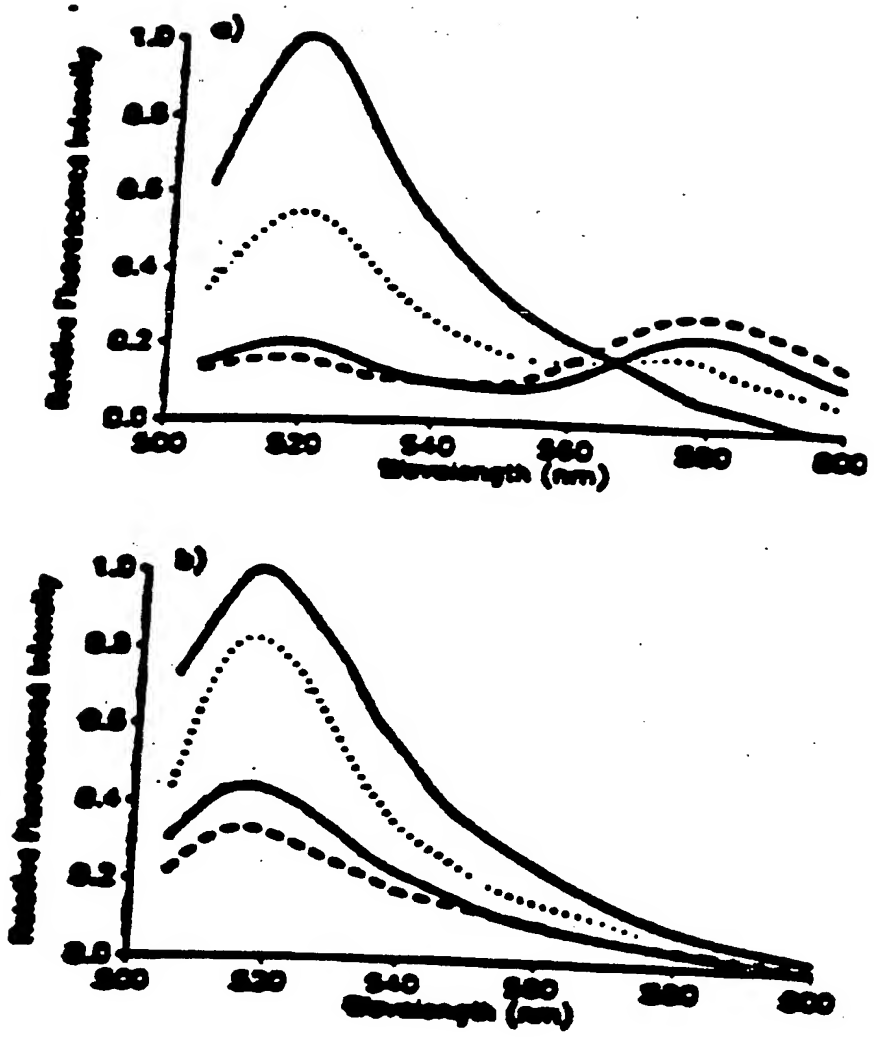


Figure 4

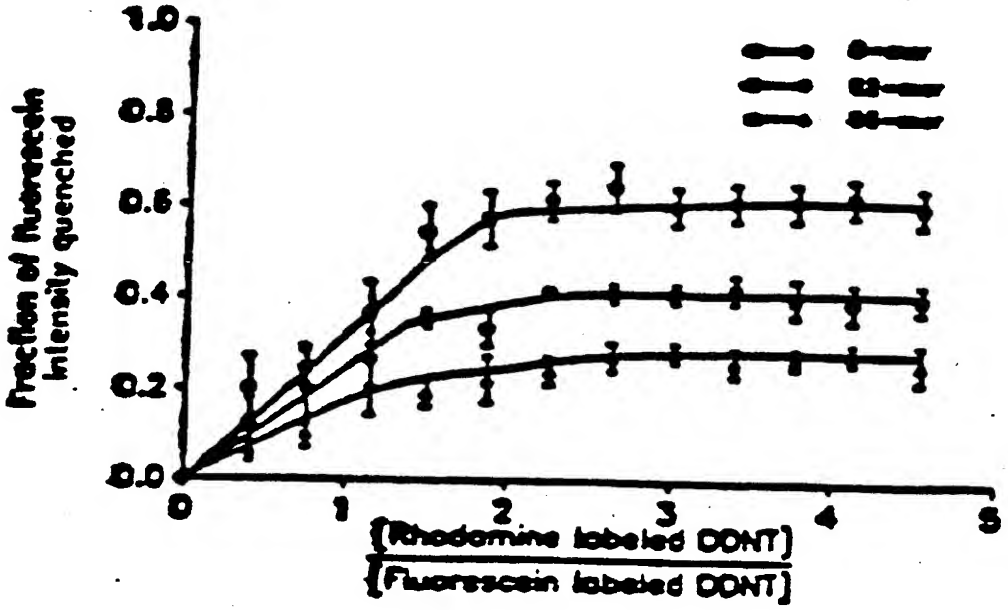

1.0
0.8
0.6
0.4
0.2
0.0
Fraction of fluorescein intensity quenched
0
1
2
3
4
5
[Rhodamine labeled DDNT]
[Fluorescein labeled DDNT]

Figure 5

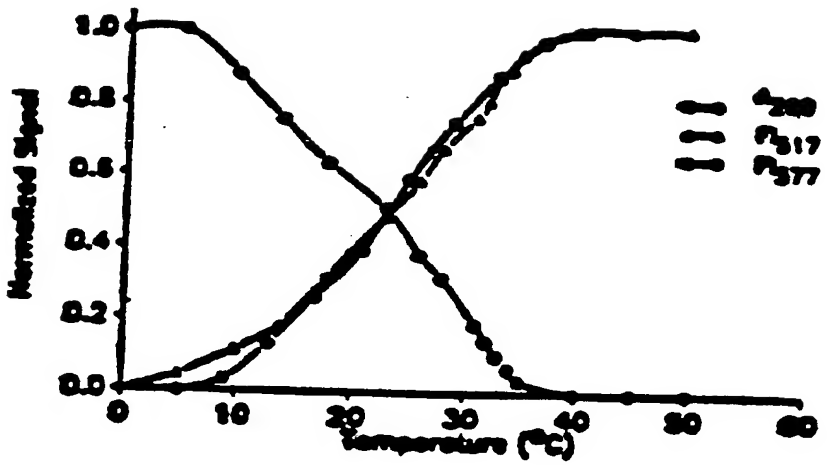


Figure 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/01591

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; G01N21/64

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X Y	WO,A,8 603 227 (DGI, INC.) 5 June 1986 see claims	5-7 1,2,4, 8-14
Y	SCIENCE. vol. 245, 8 September 1989, LANCASTER, PA US pages 1066 - 1073; J. R. RIORDAN ET AL.: 'Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA' cited in the application see the whole document	1,2,4, 8-14

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 JULY 1992	21.07.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MOLINA GALAN E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, December 1988, WASHINGTON US pages 8790 - 8794; R. A. CARDULLO ET AL.: 'Detection of nucleic acid hybridization by nonradiative fluorescence resonance energy transfer' cited in the application see the whole document	5-7
X	EP,A,0 229 943 (MOLECULAR BIOSYSTEMS) 29 July 1987 see figures	5-7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9201591 SA 58213

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 10/07/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8603227	05-06-86	US-A- 4820630	11-04-89
		AU-B- 591471	07-12-89
		AU-A- 5195786	18-06-86
		CA-A- 1258029	01-08-89
		EP-A,B 0203143	03-12-86
		JP-T- 63501051	21-04-88
EP-A-0229943	29-07-87	CA-A- 1273552	04-09-90
		DE-A- 3681272	10-10-91
		JP-A- 62157570	13-07-87
		US-A- 4996143	26-02-91

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